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Published in:
Hippocampus

DOI:
10.1002/hipo.20272

Publication date:
2007

HIPPOCAMPUS 17:338–348 (2007)

Regional Differences in Hippocampal PKA Immunoreactivity After Training and Reversal Training in a Spatial Y-Maze Task

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ABSTRACT: It is suggested that the hippocampus functions as a comparator by making a comparison between the internal representation and actual sensory information from the environment (for instance, comparing a previously learned location of a food reward with an actual novel location of a food reward in a Y-maze). However, it remains unclear to what extent the various hippocampal regions contribute to this comparator function. One of the proteins known to be crucially involved in the formation of hippocampus-dependent long-term memory is the adenosine 3′,5′ cyclic monophosphate dependent protein kinase (PKA). Here, we examined region-specific changes in immunoreactivity (ir) of the regulatory IIα,β subunits of PKA (PKA RIIα,β-ir) in the hippocampus during various stages of spatial learning in a Y-maze reference task. Thereafter, we compared changes in hippocampal PKA RIIα,β-ir induced by training and reversal training in which the food reward was relocated to the previously unrewarded arm. We show that: (1) There was a clear correlation between behavioral performance and elevated PKA RIIα,β-ir during the acquisition phase of both training and reversal training in area CA3 and dentate gyrus (DG), (2) PKA RIIα,β-ir was similarly enhanced in area CA1 during the acquisition phase of reversal training, but did not correlate with behavioral performance, (3) PKA RIIα,β-ir did not change during training or reversal training in the subiculum (SUB), (4) No changes in PKA RIIα,β protein levels were found using Western blotting, and (5) AMPA receptor phosphorylation at serine 845 (S845p; the PKA site on the glutamate receptor 1 subunit (GluR1)), was enhanced selectively during the acquisition phase of reversal training. These findings reveal that training and reversal training induce region-specific changes in hippocampal PKA RIIα,β-ir and suggest a differential involvement of hippocampal subregions in match-mismatch detection in case of Y-maze reference learning. Alterations in AMPA receptor regulation at the S845 site seems specifically related to the novelty detector function of the hippocampus important for match-mismatch detection. © **2007 Wiley-Liss, Inc.**

KEY WORDS: protein kinase A; hippocampus; learning; S845; GluR1

INTRODUCTION

One of the processes generally considered to underlie the formation and consolidation of long-term memory is activity-dependent synaptic plasticity (Bliss and Collingridge, 1993; Martin et al., 2000). A potential mechanism controlling synaptic plasticity is the balance between phosphorylation and dephosphorylation of specific substrates (Tokuda and Hatase, 1998; Soderling and Derkach, 2000; Mansuy, 2003). Studies focused on the positive regulators, revealed that protein kinases (PKA) are critically involved in learning and memory processes via enhancement of synaptic efficacy (Micheau and Riedel, 1999; Nguyen and Woo, 2003; Sweat, 2004).

A key player in the consolidation phase of long-term memory is the adenosine 3′,5′ cyclic monophosphate (cAMP)-dependent protein kinase. Upon activation, PKA can phosphorylate the glutamate receptor 1 (GluR1) serine 845 site (S845) of the AMPA receptor, resulting in enhanced incorporation of GluR1-containing AMPA receptors into the membrane (Lee et al., 2000; Esteban et al., 2003). Furthermore, activated PKA can enter the nucleus and activate the transcription factor cAMP-response element-binding protein (CREB) through phosphorylation of serine 133 (Impey et al., 1996; Bernabeu et al., 1997).

Enhanced hippocampal PKA activity was found after radial maze training (Vázquez et al., 2000) and a holeboard food search task (Mizuno et al., 2002), whereas inhibition of hippocampal PKA activity resulted in impaired long-term memory in contextual fear conditioning and water maze training (Abel et al., 1997; Wallenstein et al., 2002; Ahi et al., 2004). In addition, inhibition of PKA activity in the amygdala diminished long-term memory for conditioned taste aversion (Koh and Bernstein, 2003), while stimulation of PKA activity in the amygdala enhanced reward-related learning (Jentsch et al., 2002).

Although the importance of PKA in the formation of long-term memory has been shown using pharmacological or genetical approaches, to our knowledge, no anatomical studies have been conducted to explore whether training induces region-specific changes in hippocampal PKA immunoreactivity (PKA-ir). In this way, the importance of selective regions during a specific phase of the learning process can be shown. Previous studies have proven that immunoreactivity can be a useful tool to pick up such training induced changes (Van der Zee et al., 1997; Van der Zee and Luiten, 1999). In the first experiment of this study, we therefore investigated whether training in a reference version of a spatial Y-maze task induces region specific changes in hippocampal PKA-ir.

In addition to memory formation, it has been suggested that the hippocampus plays a key role in comparing actual sensory information from the environment with previously stored internal representations, a

Department of Molecular Neurobiology, University of Groningen, Haren, The Netherlands
Grant sponsor: The Netherlands Organization for Scientific Research (NWO-Vernieuwingsimpuls); Grant number: 016.021.017.
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Accepted for publication 22 January 2007
DOI 10.1002/hipo.20272
Published online 21 February 2007 in Wiley InterScience (www.interscience.wiley.com).

process needed to adapt to continuous environmental changes (a so called match-mismatch process) (Gothard et al., 1996; Knight, 1996). To investigate the mechanisms underlying match-mismatch processing, subjects are confronted with changes in a previously experienced context. This can be achieved by, for example, the absence of an electrical shock after learning an association between a known context and an electrical shock (referred to as extinction training) or the relocation of a hidden platform or food reward in respectively a Morris water maze, or a two arm maze (referred to as reversal training). Several studies revealed that both reversal and extinction training involve partly overlapping pathways and brain (sub)regions compared to the original training (Myers and Davis, 2002; Lin et al., 2003). Zeng et al. (2001) showed that complete disruption of calcineurin (protein phosphatase 2B) activity in the forebrain did not disrupt learning the original platform location. In contrast, learning of multiple platform locations in the Morris water maze was inhibited. Mice lacking the GluR1 subunit of the AMPA receptor displayed a mild deficit during reversal training but not during training in a spatial version of a T-maze reference task (Bannerman et al., 2003). Similar deficits in reversal training only have been found in aged rats (Stephens et al., 1985). Szapiro et al. (2003) showed that hippocampal PKA is critically involved in extinction of conditioned fear. Thus, if PKA is implicated in match-mismatch processing that is suggested to take place in the hippocampus (Gothard et al., 1996; Knight, 1996), PKA-ir may change in a region-specific way during reversal training. To test the hypothesis that PKA-ir is changed during reversal training in the Y-maze, we performed a second experiment in which we trained mice similarly to experiment 1. Afterwards, mice were received a reversal training with the food reward relocated to the previously unbaited arm. We compared the two training protocols to determine whether PKA-ir and the phoshorylation state of the GluR1 S845 site is differentially affected by training and reversal training.

MATERIALS AND METHODS

Animals and Housing Conditions

A total of 75 male C57BL/6j mice (Harlan, Horst, Netherlands), 12–15 weeks old, were individually housed in standard macrolon cages equipped with a removable slot which could be locked on to a Y-maze. Subjects were maintained on a 12 h light/dark cycle (lights on at 7.30 a.m.) with food (Hopefarm® standard rodent pellets) and water ad libitum. A layer of sawdust served as bedding. Subjects were food deprived to 90% of their individual body weight under ad libitum feeding conditions, starting 4 days before the beginning of the experiment. Animals were weighed and fed after the last session of each day.

Mice were assigned to one of the following groups: experiment 1a: home cage controls (HCC, $n = 7$); pseudotraining (PT, 3 sessions of free exploration in an unbaited maze, $n = 7$). Experiment 1b: HCC, $n = 10$; training (T3, 3 sessions of training, $n = 11$; T7, 7 sessions of training, $n = 11$). Experiment 2: reversal training (RT1, 7 sessions of training followed by 1 session of reversal training, $n = 12$; RT3, 7 sessions of training followed by 3 sessions of reversal training, $n = 9$; RT7, 7 sessions of training followed by 7 sessions of reversal training, $n = 8$). These time points were chosen to study the dynamics of PKA during both training and reversal training: acquisition phase of training (T3), end of training (T7), reversal effect (RT1), acquisition phase of reversal training (RT3), and end of reversal training (RT7).

The procedures described in the present study were approved by the Dutch Animal Experiment Committee of the University of Groningen in compliance with Dutch law and internal regulations.

Y-Maze

Behavioral testing was conducted in a tubular plexiglass Y-maze. Both start arm (27.5 cm long) and the two arms forming the Y (both 27.5 cm long and diverged at a 60° angle from the stem arm) were 5 cm in diameter. The home cage was connected to the start arm of the Y-maze. Perforations at the endings of the two arms forming the Y allowed odors from food crumbs placed next to the perforations to prevent animals to discriminate between baited and unbaited arms by olfactory cues. Each arm was equipped with a guillotine door halfway the arm which could be operated manually from the experimenters position. Small gray plastic blocks (1 cm high) were placed 4 cm from the end of the arms to prevent visual inspection for food presence from a distance. The experimental room contained visual cues, which served as distal spatial cues.

Habituation Procedure

During the first day, three habituation trials were performed (3 h interval). The first habituation trial consisted of placing the subjects in the center of the Y-maze and mice were allowed to explore the maze for 4 min. During the subsequent habituation, the home cage was connected to the start arm of the Y-maze. Mice were given the opportunity to freely enter the maze without handling. Starting from the home cage, subjects could explore one of the two arms (the other arm was closed). The open arm was baited with small crumbs of food (0.05–0.1 g) placed at the end of the arm (the PT group received the complete habituation in a unbaited maze). When the reward was consumed and the mouse retreated to the home cage, the home cage was removed from the Y-maze. The third habituation was similar to the second habituation, but now the previously blocked arm was accessible and baited, and the previously accessible arm was blocked. The second and third habituations were given to prevent the development of a preference for either of the two arms.

Test Procedure

After habituation, training sessions consisting of six trials each were given. During the entire training, either the right or left arm was baited (in case of the PT group, the maze was

unbaited). When a subject visited one of the two accessible arms, the nonvisited arm was closed. After the subject retreated to the home cage, the start arm connected to the home cage was subsequently blocked preventing re-entrance of the maze. After cleaning all arms with damped paper cloth, and re-baiting one of the two arms, the subject was again allowed to explore either the right or left arm. A visit to the baited arm was recorded as a correct trial. The T7, RT1, RT3, and RT7 group received 7 sessions of training, divided over 4 days, while the T3 group received 3 sessions of training divided over 2 days. After the training, the RT1, RT3, and RT7 group received respectively 1, 3, and 7 additional sessions in 1, 2, and 3 days, but now with the food reward located in the previously unrewarded arm (reversal training). The total number of testing days (excluding the habituation day) for the different groups: PT and T3, 3 days; T7, 4 days; RT1, 5 days; RT3, 6 days; RT7, 7 days.

Ten minutes after the last session, animals were deeply anesthetized with CO_2/O_2 followed by a quick dissection (within 2 min) of the brain. Right and left hemispheres were separated and hippocampal tissue was either processed for immunocytochemistry or biochemical analysis.

Immunocytochemistry

Hemispheres were immersion fixed in 2% paraformaldehyde at 4°C. After 18 h, hemispheres were stored in phosphate buffered saline (PBS, pH 7.4) with 0.1% sodium-azide at 5°C. Before sectioning on a cryostat, hemispheres were cryoprotected in 30% phosphate buffered sucrose for 36 h at room temperature. Coronal sections were cut at a thickness of 20 μm, collected and stored in PBS with 0.1% sodium-azide. After rinsing in PBS (3×) and incubation in 0.4% H_2O_2 (30 min) followed by rinsing in PBS (3×), sections were preincubated in 5% normal rabbit serum (Jackson immunoResearch laboratories, West Grove, PA), in PBS for 20 min at room temperature. After preincubation, sections were incubated with polyclonal goat-anti-PKA RIIα,β subunit IgG (1:1,000; Upstate Cell signaling solutions, Charlottesville, VA) in 1% normal rabbit serum and 0.1% azide in PBS at 37°C for 2 h. Incubation was continued at 5°C for 3 days. Sections were then rinsed in PBS (3×) and preincubated with 5% normal rabbit serum in PBS, followed by incubation overnight in biotinylated rabbit-antigoat IgG (1:500; Jackson ImmunoResearch laboratories, West Grove, PA) at 5°C. After rinsing with PBS (3×), sections were incubated with the avidin–biotin–horseradish peroxidase complex (1:500 ABC kit, Vector laboratories Burlingame, CA) for 2 h at room temperature. Finally after rinsing in PBS for 2 days, sections were processed with diaminobenzidine (0.02% in Tris-HCl pH 7.6) with 100 μl of 0.1% H_2O_2 as a reaction initiator.

Quantification

Total optical densities (OD) of immunostainings were measured in sections containing the dorsal hippocampus (bregma −1.94 to bregma −2.06 according to the mouse brain stereotaxic coordinates; Keith B.J. Franklin and George Paxinos, 1997, Academic press, CA). Four regions of interest were selected: DG, CA3, CA1, and SUB.

The OD is expressed in arbitrary units corresponding to gray levels using a Quantimet 550 image analysis system (Leica, Cambridge, UK). The value of background labeling was measured in the corpus callosum and extracted the OD of the area of interest, thus reducing the variability in background staining among sections. The experimenter was blind to the group assessment of individual animals during all OD measurements.

Protein Extraction

Hippocampi (weighing ∼0.017–0.020 g) were quickly dissected on an ice-cold block and homogenized in ice-cold lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM β-mercaptoethanol, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 10 mM benzamidine, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 50 mM NaF, and an inhibitor cocktail (added right before use, Roche, Mannheim, Germany) using a pellet pestle (Sigma-Aldrich, St. Louis, MO). The resulting homogenates were centrifuged for 50 min at 100,000*g* at 5°C. The high-speed supernatants were collected for use as cytosol fractions. The high-speed pellets, containing plasma membrane and pellet membrane-bound subcellular organelles, including nuclei, mitochondria, microsomes (Hunzicker-Dunn and Jungman, 1978), synaptosomes (Kikkawa et al., 1983), and cytoskeletal components (Kiley and Jaken, 1990), were resuspended in lysis buffer and the resulting suspensions were used as pellet fractions. The protein concentrations were measured using the Bradford method (Bradford, 1976). Subsequently SDS sample buffer (50% glycerin, 321.5 mM Tris/HCl pH 6.8, 10% SDS, 25% β-Mercaptoethanol, 0.1% Bromophenol Blue) was added, followed by 5 min heat denaturation at 95°C. Afterwards samples were stored at −80°C.

Western Blotting

Ten microgram of protein were separated on 10% SDS-polyacrylamide gels, blotted electrophoretically to Immobilon-P transfer membrane (Millipore, Bedford, MA) and blocked for 1 h in blocking buffer (0.1% Tween-20, 0.2% I-block, Tropix, Bedford, MA, in PBS) at 5°C. For detection of protein levels of PKA regulatory subunits or, membranes were incubated with either polyclonal goat-anti-PKA RIIα,β subunit IgG (1:1,000; Upstate Cell signaling solutions, Charlottesville, VA), polyclonal rabbit-antiphospho serine 845 IgG (1:2,000; Upstate Cell signaling solutions, Charlottesville, VA), or polyclonal rabbit-anti-GluR1 (1:20,000; Upstate Cell signaling solutions, Charlottesville, VA) in buffer (containing 0.05% Tween-20, 0.1% I-block, Tropix, Bedford, MA, in PBS) overnight at 5°C. Monoclonal mouse-antiactin IgG antibody (clone C4, MP biomedicals, Irvine, CA) was used to correct for protein levels. Membranes were rinsed with blocking buffer (2×) and incubated with the proper secondary antibodies (1:10,000, alkaline phosphatase conjugated donkey-antigoat, Santa Cruz Biotechnology, Santa Cruz, CA, 1:10,000 alkaline phosphatase conjugated goat-antimouse, Tropix, Bedford, MA, USA or 1:10,000 alkaline phosphatase conjugated goat-antirabbit, Tropix) in

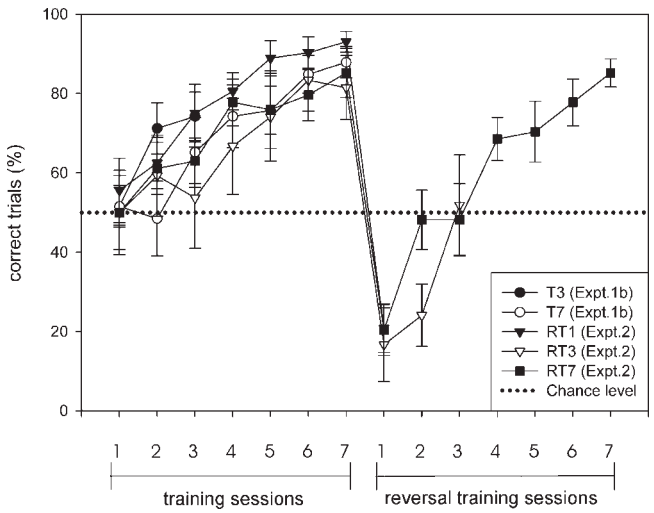

FIGURE 1. Performance in the Y-maze during acquisition training and reversal training (Experiment 1 and 2 combined). The percentage of correct trials per session is shown for the T3 ($n = 11$), T7 ($n = 11$), RT1 ($n = 12$), RT3 ($n = 9$), and RT7 ($n = 8$) group.

buffer (containing 0.05% Tween-20, 0.1% I-block, Tropix in PBS) for 30 min at room temperature. Following rinsing with blocking buffer (2×), membranes were rinsed in assay buffer (0.1 M diethanolamine, 1 mM $MgCl_2$, pH 10.0) for 2 × 5 min at RT. For chemoluminescent labeling, membranes were incubated with Nitroblock II (1:40 in assay buffer, Tropix), rinsed with assay buffer (2×) and finally incubated with CDP star substrate (1:1,000, Tropix) in assay buffer for 5 min at room temperature.

The immunoreactive bands were captured on autoradiography film (Kodak X scientific image film, Rochester, NY). Densitometric scans of the immunoreactive bands were digitized and quantified using a Quantimet 500 image analysis system (Leica, Cambridge, UK).

Statistical Analysis

Analysis of the behavioral data was performed using repeated measures analysis. The ODs of the immunocytochemistry data were analyzed with one way analysis of variance (ANOVA) followed by post hoc comparisons using a Dunnett test. Pearson correlations were used to analyze the relationship between behavioral performance and ODs. $P < 0.05$ was considered as significant.

RESULTS

Behavioral Performance in the Y-Maze During Training and Reversal Training

During training in the Y-maze, all groups gradually increased their performance ($F_{6,216} = 29{,}238$ $P < 0.001$; group and interaction $F < 1$ in both cases; Fig. 1), resulting in a final score ranging from 81.5% (±8.0%) to 93.1% (±2.6%) after 7 sessions. After training, the RT1, RT3, and RT7 groups were confronted with a relocation of the food reward to the previously unbaited arm (reversal training). The performance dropped to a score ranging from 16.7% ± 9.3% to 20.8% ± 6.1% correct trials after one session of reversal training, demonstrating that mice still had a preference for the previously rewarded arm. Gradually, mice shifted their preference to the previously unrewarded arm and reached the same level of performance as was found during training (R7 group, 81.25% ± 7.99%; $F_{6.42} = 23{,}837$ $P < 0.001$; Fig. 1).

PKA RIIα,β-ir in the Dorsal Hippocampus of HCC Animals

PKA RIIα,β-ir in brains sections of HCCs revealed a characteristic pattern in granular cells, pyramidal cells, and nonpyramidal cells throughout the dorsal hippocampus (Fig. 2). This characteristic pattern was in agreement with previously reported hippocampal distribution of PKA RIIα,β (De Camilli et al., 1986; Ludvig et al., 1990). The most pronounced staining was found in the CA3 pyramidal cell bodies and apical dendrites (OD: 0.274 ± 0.053 and 0.208 ± 0.037, respectively). Compared to the CA3 area, pyramidal cell bodies of the SUB and CA1 area (OD: 0.065 ± 0.011 and 0.052 ± 0.010, respectively) and their dendrites (OD: 0.036 ± 0.009 and 0.034 ± 0.009, respectively) were moderately stained. The granular cell bodies in the DG showed the weakest labeling (OD: 0.026 ± 0.008), while the dendrites were moderately stained (OD: 0.11 ± 0.014). Interneurons of the Hilus were intensely stained (OD not measured).

Changes in PKA RIIα,β-ir in the Dorsal Hippocampus Induced by Pseudotraining (Experiment 1a)

Mice that received 3 sessions free exploration in an unbaited Y-maze did not show any preference for either of the two acces-

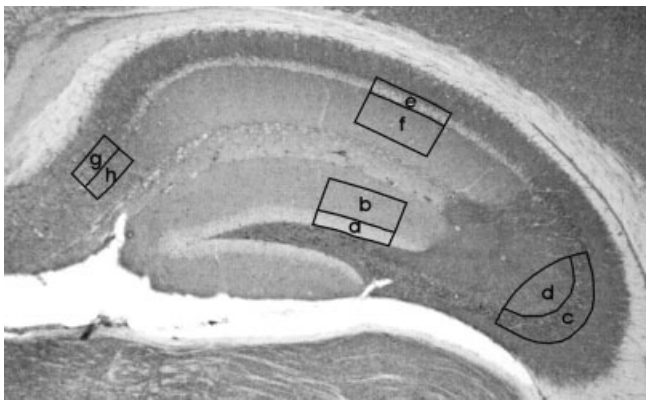

FIGURE 2. Distribution of PKA RII α,β-ir in the dorsal hippocampus. The delineated regions indicate the for optical density analyzed areas of cell bodies and dendrites of the granular cells (a and b), CA3 pyramidal cells (c and d), CA1 pyramidal cells (e and f), and SUB pyramidal cells (g and h).

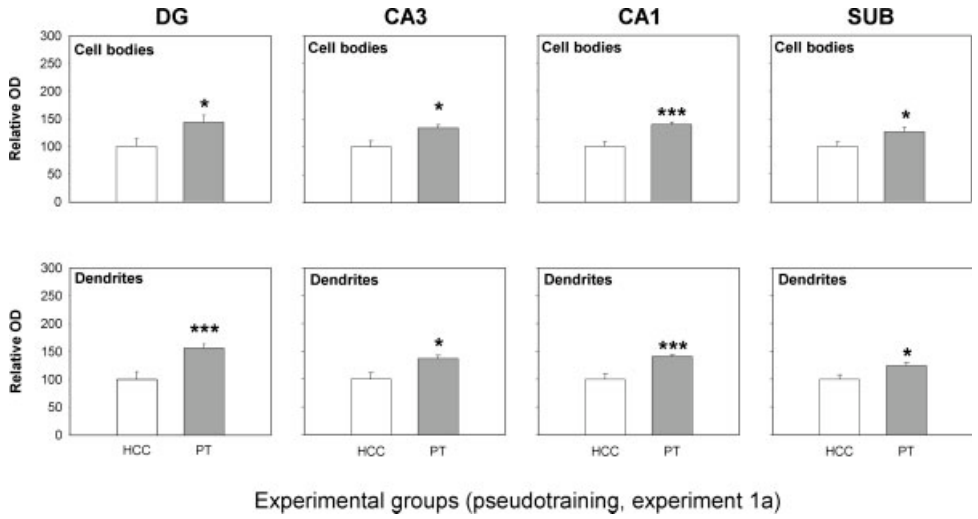

FIGURE 3. Three sessions of pseudotraining increased PKA RIIα,β-ir in the dorsal hippocampus. PKA RIIα,β-ir was measured in the following areas of the dorsal hippocampus of the PT group and compared to the HCC group: DG, CA3, CA1, and SUB in both cell bodies and dendrites. *$P < 0.05$, ***$P < 0.005$.

sible arms (data not shown). Afterwards, we assessed whether exploration in an unbaited Y-maze was sufficient to change hippocampal PKA RIIα,β-ir. Pseudotraining in an unbaited Y-maze enhanced PKA RIIα,β-ir in all measured hippocampal areas compared to the HCC group (Fig. 3). Strongest increases in PKA RIIα,β-ir were found in the DG granular dendrites ($P < 0.005$) and in the CA1 area (pyramidal cell bodies $P < 0.005$, dendrites $P < 0.005$). Moderate increases in PKA RIIα,β-ir were found in the DG granular cell bodies ($P < 0.05$), CA3 pyramidal cell bodies and dendrites (in both cases $P < 0.05$), and SUB pyramidal cell bodies and dendrites (in both cases $P < 0.05$).

Changes in PKA RIIα,β-ir in the Dorsal Hippocampus Induced by Training (Experiment 1b)

Next, we explored whether training in a baited Y-maze increased PKA RIIα,β-ir in the dorsal hippocampus. Compared to the HCC group, PKA RIIα,β-ir was significantly increased in the CA3 area (both pyramidal cell bodies and dendrites $P < 0.01$, Fig. 4) and dendrites of the granular cells in the DG ($P < 0.005$) after three sessions of training, when an average performance of 80% correct trials per session was not yet reached (T3 group). The enhanced PKA RIIα,β-ir in both

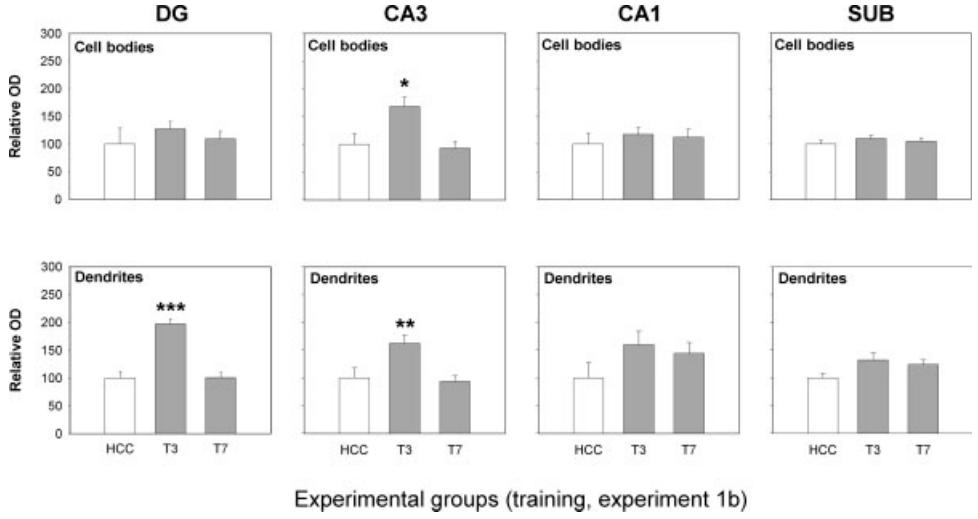

FIGURE 4. Training in the Y-maze (T3 group) enhances PKA RIIα,β-ir in the granular cell dendrites and CA3 pyramidal cells (cell bodies and dendrites). PKA RIIα,β-ir was measured in the following areas of the dorsal hippocampus of the T3 and T7 group and compared to levels of the HCC group: DG, CA3, CA1, and SUB in both cell bodies and dendrites. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

TABLE 1.

Correlations Between OD Measures for PKA RIIα,β-ir in the Hippocampus and Behavioral Performance in the Y-Maze

Area	Group	T3	RT3
DG	Cell bodies	0.341	0.098
	Dendrites	0.919**	0.788**
CA3	Cell bodies	0.656*	0.817*
	Dendrites	0.669*	0.854*
CA1	Cell bodies	0.059	0.167
	Dendrites	0.215	−0.067

Enhanced hippocampal PKA RIIα,β-ir in CA3 pyramidal cells and dendrites of granular cells is positively correlated with behavioral performance during training and reversal training. R^2 values are shown.
*$P < 0.05$,
**$P < 0.01$.

regions correlated positively with the behavioral performance (Table 1: CA3 cell bodies and dendrites $P < 0.05$, DG dendrites $P < 0.01$). At the end of traning, when an average performance of 80% correct trials per session was reached (T7 group), PKA RIIα,β-ir returned to baseline values in these areas. No increase in PKA RIIα,β-ir was found in other regions of the hippocampus ($P > 0.1$ for all comparisons).

Changes in PKA RIIα,β-ir in the Dorsal Hippocampus Induced by Reversal Training (Experiment 2)

Thereafter, we assessed whether reversal training in a baited Y-maze changed PKA RIIα,β-ir in regions of the dorsal hippocampus. After 1 session of reversal training (RT1 group), PKA RIIα,β-ir was enhanced in the dendrites of CA3 pyramidal cells ($P < 0.05$, Fig. 5) and dendrites of the granular cells ($P < 0.005$, Fig. 5). After 3 sessions of training, when an average performance of 80% correct trials per session was not yet reached (RT3 group), PKA RIIα,β-ir was increased in both cell bodies and dendrites of CA3 pyramidal cells ($P < 0.005$) and granular cell dendrites ($P < 0.005$). Enhanced levels correlated positively with behavioral performance of the RT3 group (Table 1: CA3; cell bodies and dendrites $P < 0.05$, DG; dendrites $P < 0.05$). In area CA1, PKA RIIα,β-ir was also increased after 3 sessions of reversal training (RT3 group) in both CA1 cell bodies ($P < 0.01$, Fig. 5) and dendrites ($P < 0.005$, Fig. 5). In contrast to DG and CA3, elevated PKA RIIα,β-ir levels in the CA1 area of the R3 group did not correlate with behavioral performance. All enhanced PKA RIIα,β-ir levels returned to baseline values at the end of reversal training, when an average performance of 80% correct trials per session was reached (RT7 group, Fig. 5). No changes were found in the SUB pyramidal cells ($P > 0.05$, Fig. 5).

PKA RIIα,β Immunoblots of the Hippocampus

To determine whether training and reversal training induced changes in protein levels of PKA RIIα,β, Western blot analysis on whole hippocampi were performed on the pellet fraction. This fraction contains the majority of PKA RIIα,β subunits (Pawson and Scott, 1997; Nguyen and Woo, 2003). Figure 6A shows a representative blot for PKA RIIα,β. Immunoreactive bands were found at a size of 53 kD which corresponds to the size of the RIIα,β subunit of PKA. The IODs of the immunoreactive bands are shown in Figure 6B. No changes in protein levels of PKA RIIα,β were found after training and reversal training (ANOVA $F < 1$).

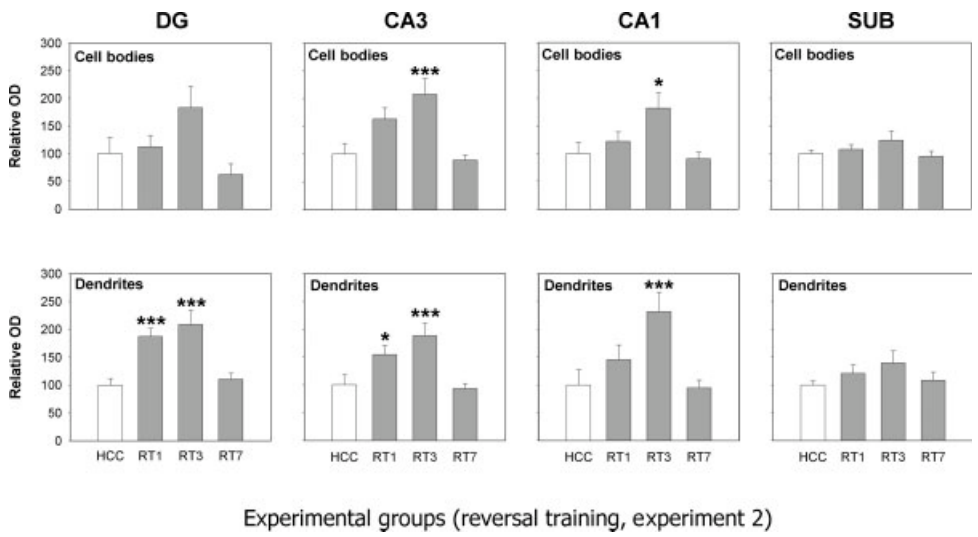

FIGURE 5. Hippocampal PKA RIIα,β-ir is enhanced in the granular cell dendrites, CA3 pyramidal cells (cell bodies and dendrites), and CA1 pyramidal cells (cell bodies and dendrites) during the acquisition phase of reversal training (group RT1 and RT3). PKA RIIα,β-ir was measured in the following areas of the dorsal hippocampus of the RT1, RT3, and RT7 group and compared to levels of the HCC group: DG, CA3, CA1, and SUB in both cell bodies and dendrites. Note that the same HCC group was used to analyze the enhanced PKA RIIα,β-ir induced by training and reversal training. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$.

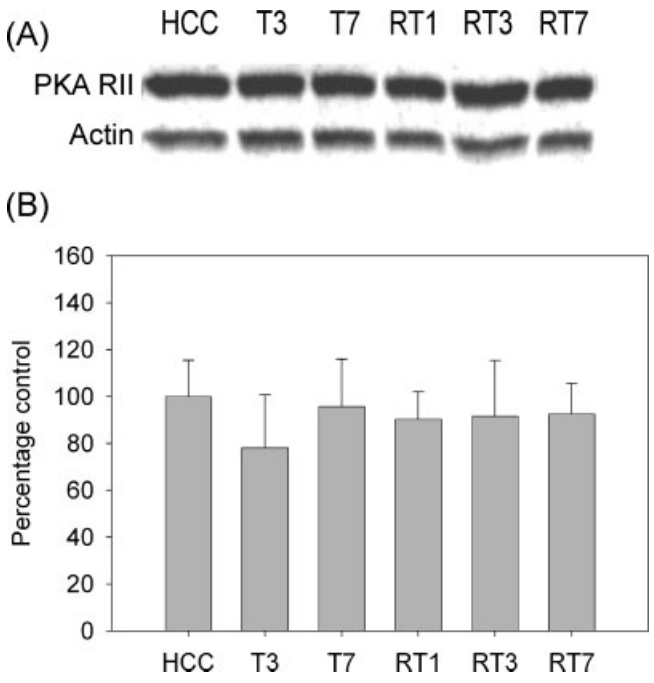

FIGURE 6. Hippocampal PKA RIIα,β protein levels in the pellet fraction were not changed during training or reversal training in the Y-maze. (A) Representative immunoreactive bands for PKA RIIα,β and actin. (B) Quantification of hippocampal PKA RIIα,β protein levels, relative to actin levels for all groups.

S845p Immunoblots of the Hippocampus

Finally, we assessed whether changes in PKA RIIα,β-ir during training and reversal training were accompanied by changes in serine 845 phosphorylation (S845p) of the PKA site on GluR1. Figure 7A shows a representative blot for S845p. Immunoreactive bands were found at a size of ~106 kD which corresponds to the size of the serine 845 site of the GluR1 subunit. The IODs of the immunoreactive bands are shown in Figure 7B. No changes in S845p were found during training. However, S845p levels were significantly enhanced after three sessions of training, when an average performance of 80% correct trials per session was not yet reached (RT3 group, $P < 0.05$). S845p levels returned to baseline values at the end of reversal training, when an average performance of 80% correct trials per session was reached (RT7 group, Fig. 7B). No changes in GluR1 protein levels were found (ANOVA $F < 1$).

DISCUSSION

In this study, we investigated whether pseudotraining, training, and reversal training in a Y-maze induced changes in hippocampal PKA RIIα,β-ir and PKA RIIα,β protein levels and whether this was accompanied by changes in GluR1 S845p. We show that: (1) PKA RIIα,β-ir is enhanced in all regions of the hippocampus after pseudotraining in an unbaited Y-maze, (2) PKA RIIα,β-ir is enhanced and correlated positively with behavioral performance during the acquisition phase of both training (T3 group) and reversal training (RT1 group and RT3 group) in CA3 pyramidal cells (cell bodies and dendrites) and dendrites of the DG granular cells, (3) PKA RIIα,β-ir was increased in area CA1 during the acquisition phase of reversal training only, but did not correlate with behavioral performance, (4) PKA RIIα,β-ir was not changed during training or reversal training in the SUB pyramidal cells (cell bodies and dendrites) or in the cell bodies of the granular cells in the DG, (5) no changes in PKA RIIα,β protein levels were found in the pellet fraction, and (6) S845p was enhanced only during the acquisition phase of reversal training (RT3 group).

In general, these results are in line with the widely accepted view that hippocampal PKA is involved in memory formation and consolidation (Abel et al., 1997; Bourtchouladze et al., 1998; Vázquez et al., 2000; Mizuno et al., 2002; Wallenstein et al., 2002; Nguyen and Woo, 2003; Ahi et al., 2004), and showed that pseudotraining, training, and reversal training in a Y-maze reference task differentially affect hippocampal PKA RIIα,β-ir as well as S845p of the GluR1 subunit of the AMPA receptor.

Changes in Hippocampal PKA RIIα,β-ir Induced by Pseudotraining

Three sessions of free exploration in an unbaited maze resulted in an overall enhanced PKA RIIα,β-ir in the dorsal hippocampus ranging from a 56% increase in the dendrites of

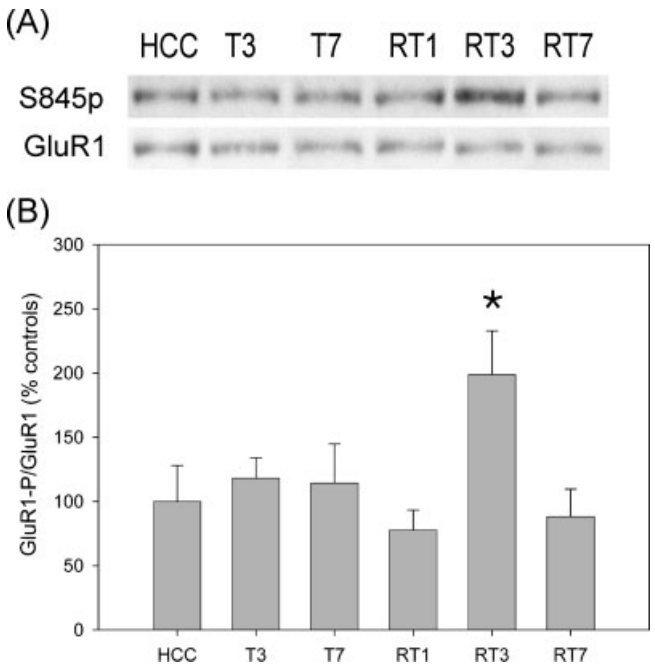

FIGURE 7. Hippocampal S845p levels are enhanced selectively during the acquisition phase of reversal training (RT3 group). (A) Representative immunoreactive bands for S845 and GluR1. (B) Quantification of hippocampal S845p levels, relative to GluR1 levels for all groups. *$P < 0.05$.

the DG granular cells to a 24% increase in the dendrites of the SUB pyramidal cells. In line with our findings, previous studies showed that pseudotraining in a context resulted in an overall enhanced staining in all immunoreactive neurons in the entire mouse hippocampus for the gamma isoform of protein kinase C (PKCγ, Van der Zee et al., 1992), as well as for the muscarinic acetylcholine (ACh) receptor (Van der Zee et al., 1995). Together, these results indicate that task-specific information not related to a reward leads to enhancement of PKA RIIα,β-ir in a nonspecific manner. This pattern of enhanced expression induced by pseudotraining could however be involved in the initial storage of spatial information concerning the context of the Y-maze.

Changes in Hippocampal PKA RIIα,β-ir Induced by Training (Group T3 and T7)

The importance of the CA3 area in the acquisition phase of training, in which contextual representations are formed, has been shown previously (Gall et al., 1998; Kelly and Deadwyler, 2002; Lee and Kesner, 2004; Daumas et al., 2005). Besides area CA3, also the DG is implicated in the formation of contextual representations generated in the CA3 area (for review see Rolls and Kesner, 2006), through the orthogonalization of similar entorhinal input patterns and amplifying differences in these patterns before they are sent to the CA3 region (Treves and Rolls, 1994; Lee and Kesner, 2004). Thus, it could be that the enhanced PKA RIIα,β-ir in DG and CA3 area during the acquisition phase of training, reflects enhanced activity of these two regions involved in the encoding of representations of the context of the maze as well as a representation of the location of the food reward within the maze, which are both needed to make correct responses in the Y-maze learning paradigm.

At the end of training, PKA RIIα,β-ir returned back to levels comparable to those of control mice. The return of immunoreactivity to baseline levels suggests that, at least in case of PKA, the CA3-DG complex is specifically implicated in the acquisition phase of training in the Y-maze. The importance of the CA3-DG complex specifically in the acquisition phase of training has been shown previously for other learning paradigms (Handelmann and Olton, 1981; Gall et al., 1998; Vázquez et al., 2000).

Changes in Hippocampal PKA RIIα,β-ir Induced by Reversal Training (Group RT1, RT3, and RT7)

After 1 session of reversal training, where subjects were confronted with the relocation of the food reward for the first time (i.e. the reversal effect), PKA RIIα,β-ir was increased specifically in the dendrites of the granular cells and dendrites of CA3 pyramidal cells. In line with this finding, Lee et al. (2004) showed that when rats encountered changes for the first time in a previously experienced environment, area CA3 reacts as first to environmental changes if these changes are significant. This could explain why the reversal effect resulted in an immediate enhancement of PKA RIIα,β-ir in the DG and CA3 dendrites.

During the following reversal training sessions, PKA RIIα,β-ir was not only enhanced in the dendrites of the granular cells and CA3 pyramidal cells, but now also in CA3 pyramidal cell bodies as was found in the acquisition phase of Y-maze training (T3 group). Enhanced expression patterns in DG and CA3 area (in the RT3 group) were positively correlated with behavioral performance as was found during Y-maze training. Reenhancement of PKA RIIα,β-ir suggests that PKA might be involved in the encoding of a new memory trace, that incorporates the novel location of the food reward leading to a changed behavioral response pattern increasing preference for the newly baited arm.

Interestingly, elevated PKA RIIα,β-ir was found in the CA1 area during the acquisition phase of reversal training (RT3 group). In contrast to DG and CA3, increased PKA RIIα,β-ir in CA1 area did not correlate with the behavioral performance. This suggests that the elevated PKA RIIα,β-ir in this area is a consequence of the relocation of the food reward rather than the behavioral performance. Thus, it could be that CA1 PKA RIIα,β-ir is enhanced due to the mismatch between retrieved information independent of (derived from the entorhinal cortex (EC)) and actual experience (processed by the DG-CA3 complex). In line with this interpretation, Fyhn et al. (2002) showed that CA1 firing patterns are changed when there is a mismatch between the expected and actually experience platform location in a water maze task. Furthermore, enhanced levels of Fos protein were found in the CA1 area after relocation of previously presented familiar items (Jenkins et al., 2004; Wan et al., 1999). As was found at the end of training, PKA RIIα,β-ir levels returned to baseline levels in all regions at the end of the reversal training, suggesting that hippocampal PKA plays a minor role when subjects have acquired the new location of the food reward.

Comparison of Changes in Hippocampal PKA RIIα,β-ir Induced by Training and Pseudotraining

Three sessions of free exploration in an unbaited maze induced an overall increase in PKA RIIα,β-ir in the hippocampus. In contrast, 3 sessions of training in a baited Y-maze selectively increased PKA RIIα,β-ir in DG granular cell dendrites and CA3 pyramidal cells. Interestingly, the increases in PKA RIIα,β-ir were considerably stronger during training (DG, 100%; CA3, 65%), than during pseudotraining (DG, 55%; CA3, 35%) in the Y-maze. These results show that pseudotraining and training in the Y-maze differentially affect PKA RIIα,β-ir in the dorsal hippocampus as has been previously shown for PKCγ (Van der Zee et al., 1992).

Hippocampal PKA RIIα,β Protein Levels are not Changed During Training or Reversal Training

Although we found region-specific changes in PKA RIIα,β-ir induced by training and reversal training, we did not find any changes in PKA RIIα,β protein levels in the pellet fraction induced by training or reversal training. This fraction contains

the vast majority of PKA RIIα,β subunits (due to targeting of PKA to particular substrates by anchoring proteins, scaffolding proteins, or adaptor proteins (Pawson and Scott, 1997)). The lack of changes in PKA RIIα,β protein levels induced by training and reversal training could be due to our sampling procedure. Analysis of PKA RIIα,β protein levels were performed on whole hippocampal lysates, whilst the changes in PKA RIIα,β-ir were analyzed in detail only in the dorsal part of the hippocampus. Thus, if Y-maze training would induce opposite effects on PKA RIIα,β-ir in the dorsal and ventral part of the hippocampus, this could result in no absolute change in PKA RIIα,β protein levels in the whole hippocampus. However, preliminary results revealed no opposite changes in PKA RIIα,β-ir in the ventral part compared to the dorsal part of the hippocampus. Alternatively, the enhanced PKA RIIα,β-ir does not have to be a result from increased PKA RIIα,β protein levels. In line with this suggestion, Mizuno et al. (2002) showed that radial maze training did not induce any changes in hippocampal protein levels of PKA RIIα,β subunits but rather affects the phosphorylation state of the protein. Thus, it could be that the experimentally induced changes in PKA RIIα,β-ir is due to conformational changes of the protein, for instance a change in the activity state of the protein, resulting in an increased accessibility of the epitope. Similar results were found for other proteins kinases like PKCγ (Van der Zee et al., 1997; Van der Zee and Luiten, 1999). Future experiments using phosphorylation specific PKA RII antibodies together with region-specific analysis of hippocampal PKA RIIα,β protein levels should further clarify whether enhancement of PKA RIIα,β-ir induced by Y-maze training and reversal training is a result of increased PKA RIIα,β protein levels and/or due to a conformational change of the PKA RIIα,β protein related for instance to a change in phosphorylation state.

Hippocampal GluR1 Phosphorylation at Serine 845 is Enhanced During Reversal Training Only

We assessed whether changes in PKA RIIα,β-ir were indicative for enhanced PKA activity by studying the level of AMPA receptor phosphorylation at the serine 845 site (a target of PKA, Lee et al., 2000; Esteban et al., 2003). During the acquisition phase of reversal training (RT3 group), we found strong increases in PKA RIIα,β-ir in the DG, CA3, and CA1 area. At the end of the reversal training, PKA RIIα,β-ir returned to baseline levels. This was indeed mimicked by a strong increase in S845p, with a peak during the acquisition phase of reversal training and a return to baseline values at the end of reversal training. A less pronounced increase in PKA RIIα,β-ir (in area CA3 as well as in granular cells dendrites, but not in area CA1) was found during Y-maze training. However, this was not accompanied by a change in S845p. The modest increase in PKA RIIα,β-ir in the RT1 group, reflecting the reversal effect, also did not induce a change in S845p.

Functionally, an increase in S845p is suggested to lead to increased incorporation of GluR1-containing AMPA receptors into the membrane (Lee et al., 2000; Esteban et al., 2003), whereas removal of integrated AMPA receptors has been associated with a decrease in S845p (Lee et al., 2000; Smith et al., 2006). This incorporation most likely reflects enhanced synaptic plasticity, and our results suggest therefore that the hippocampus is strongly activated during the acquisition phase of reversal training (e.g. in the RT3 group). As mentioned earlier, novelty detection (in our case the relocation of the food reward) was accompanied by a strong increase in PKA RIIα,β-ir in area CA1. Therefore we expect that enhanced S845p during the acquisition phase of reversal training is primarily located in area CA1. Future research analyzing S845p for selective subregions of the hippocampus should clarify this point.

The lack of changes for S845p during acquisition training is in line with other studies. One trial passive avoidance training was not accompanied by enhanced S845p (Bevilaqua et al., 2005; Whitlock et al., 2006). These results suggest that during training, PKA does not target this site resulting in enhanced S845p. If enhanced PKA RIIα,β-ir does reflect enhanced PKA activity, the most likely explanation is that another substrate is targeted by PKA. This is also suggest by our data of calcineurin activity (Havekes et al., 2006), the phosphatase that dephosphorylates GluR1 at S845. Calcineurin activity is reduced during Y-maze training, but this does not lead to enhanced S845p which could be the case if PKA continued to target GluR1 at S845. At the end of reversal training, GluR1 S845p levels are back to baseline, most likely due to calcineurin dephosphorylating this site. Indeed, calcineurin activity is no longer reduced at this time point.

Overall Conclusion

Taken together, our data shows that pseudotraining (to some extent), training and most notably reversal training in the Y-maze induce region-specific changes in PKA RIIα,β-ir patterns in the hippocampus. During reversal training, this is accompanied by enhanced levels of S845p of the GluR1 AMPA receptor subunit. Our findings suggest that hippocampal PKA has a region-specific function in relation to memory formation and match-mismatch detection in a previously experienced environment. Alterations in AMPA receptor regulation at the S845 site seems specifically related to match-mismatch detection of the hippocampus.

Acknowledgments

We thank Ingrid M. Nijholt for critically reading the manuscript and Anniek K.D. Visser for her technical assistance.

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